mg/kg). During the treatment period body weight and 24-hour food intake measurements were recorded daily for 13 days.

Results

1-20-PEG Ax-15 treatment reduced body weight in DIO mice in a dose-dependent manner (Figure 21). At 0.7 mg/kg, 1-20-PEG Ax-15 caused a nearly 32% weight loss, where as non-pegylated Ax-15 at the same dose decreased body weight by only 8%. In addition, weight loss was closely correlated to a decrease in food intake, with the greatest loss of appetite observed in the high dose (0.7 mg/kg 1-20-PEG Ax-15) treatment group (Figure 22). The duration of appetite suppression was longest in this treatment group as well (Figure 22). These findings suggest that pegylation enhances the efficacy of Ax-15 in reducing body weight in DIO mice by 4-fold (Figure 21). Thus, pegylation of Ax-15 may allow for lower doses and less frequent dosing regimens.

Example 16: The use of Ax-15 to treat non-insulin dependent Diabetes Mellitus (NIDDM).

Background

Non Insulin Dependent Diabetes Mellitus (NIDDM or Type II diabetes) affects about 5% of the population and is characterized by elevated blood glucose which arises primarily due to resistance to insulin's action in peripheral tissue. NIDDM is one of the most common metabolic diseases and is determined by both environmental and genetic factors. Attempts to uncover the molecular identity of specific NIDDM susceptibility genes has led to the identification of

94

several abnormalities which may contribute to the disease in small subsets of individuals. However, the molecular identity of the genes involved in the most common, late-onset form of NIDDM have yet to be identified.

5   C57BL/KsJ *db/db* (*db/db*) mice are the best studied animal model of NIDDM. These mice are insulin resistant and also exhibit a myriad of metabolic and hormonal abnormalities such as massive obesity, hyperphagia, and low energy expenditure (Kodama, H., et al., 1994 Diabetologia 37:739-744). In *db/db*, as well as in human

10   NIDDM, there is a diminished homeostatic control of glucose metabolism, highlighted by high plasma glucose levels as well as delayed glucose disappearance as evaluated by oral glucose tolerance testing (OGTT). Systemic administration of ciliary neurotrophic factor (CNTF) is known to reduce the obesity in mice which lack

15   either functional leptin (ob/ob mice) or the leptin receptor (*db/db* mice) (Gloaguen, I. et al., 1997, Proc Natl Acad Sci 94:6456-6461). Our studies with this model have shown a dramatic effect of Ax-15 treatment on food intake and bodyweight regulation (described in detail *infra*), as well as a dramatic effect on glucose tolerance,

20   which can not be ascribed to weight loss alone. Treatment of animals for 10 days with Ax-15 significantly improves the oral glucose profile in a dose-related fashion as compared to pair-fed and vehicle-treated diabetic mice (described in detail *infra*). This suggests an improvement in the animal's ability to dispose of an

25   injected glucose bolus either in an insulin-dependent or insulin-independent manner. Importantly, fasting plasma glucose and insulin levels (described in detail *infra*) are significantly reduced to near normal, non-diabetic levels in mice treated with Ax-15. As there is

95

a strong correlation between a high fasting serum insulin levels and insulin resistance in NIDDM, these results suggest that Ax-15 treatment produces a significant reduction in insulin resistance in this experimental model. There is also a significant reduction in free fatty acid levels in Ax-15-treated mice vehicle-treated control db/db mice (described in detail infra).

These combined data suggests that Ax-15 treatment results in an improvement in disposal of glucose and an increased sensitivity to insulin, which can not be attributed to decreased food intake and consequent weight loss. At a biochemical level it is known that insulin signaling involves a cascade of events initiated by insulin binding to its cell surface receptor, followed by autophosphorylation and activation of receptor tyrosine kinases, which result in tyrosine phosphorylation of insulin receptor substrates (IRSs) (Avruch, J., 1998, Molecular Cell Biochem 182:31-48). While the majority of insulin's action is thought to be mediated by its receptors in the periphery, it is also known that neurons in the arcuate nucleus express the insulin receptor and IRSs (Baskin, D.G., et al., 1993, Reg Peptides 48:257-266; Schwartz, M.W., et al., 1992, Endocr Rev 13:387-414). Our assessment of p(tyr) (pTyr) staining proteins in the arcuate nucleus of db/db animals surprisingly revealed constitutive activation of proteins, presumably IRSs, when compared to heterozygous litter mates (db/?). This aberration is attenuated by both Ax-15 doses tested in these experiments and suggests restoration of normal signaling to the insulin signaling pathway in this region.

Another well defined action of insulin is the binding of IRSs to the regulatory subunit of phosphoinositide (PI) 3-kinase, which has

96

been shown to be necessary for many of insulin actions (glucose transport, protein synthesis, and glycogen synthesis) (Shepard, P.R., et al., 1996, J. Mol Endocr 17:175-184.). The only PI3-kinases that are currently known to be stimulated by insulin are the class I

5   heterodimeric p85/p110 catalytic PI3 kinases. The p85 subunit acts as an adaptor which links the p110 catalytic subunit to the appropriate signalling complex. All of the forms of this adaptor subunit contain SH2 domains which bind to tyrosine phosphorylated motifs on IRS-1, IRS-2, and growth factor receptors (see Shepard,

10   ibid.). Analysis of liver tissue from Ax-15-treated *db/db* mice reveals a restoration of the ability of insulin to promote p85 association with p(Tyr) proteins in response to insulin. These combined results suggest that Ax-15 treatment can (1) improve the ability of *db/db* animal to dispose of glucose and (2) that

15   assessment of individual tissues suggests an increased sensitivity to insulin.

The object of this study was to characterize the effects of Ax-15, a modified CNTF, on the diabetic profile in the *db/db* mouse model of NIDDM.

20

Experimental Procedures

(1) Animals

Male *db/db* C57BL/KsJ mice (Jackson Laboratories, Bar Harbor, ME), aged 6-8 weeks, were housed in a room maintained at 69-75oC

25   with lights on for 12 hours per day. All animal procedures were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC). Starting at 10 weeks of age, mice were individually housed, received standard mouse chow

97

(Purina Mills, Richmond, IN) *ad libitum* and had free access to water. "Pair-fed" animals were provided with the same amount of food on a daily basis as the average amount ingested by the highest dose of Ax-15 in all studies reported. Ax-15 (0.1 and 0.3 mg/kg, s.c.) and

5   vehicle (10 mM Sodium Phosphate, 0.05% Tween 80, 3% PEG 3350, 20% Sucrose pH 7.5) were injected daily at approximately the same time each day. Animal body weights were recorded daily and, where indicated, blood samples collected from tail veins into capillary tubes. For an oral glucose tolerance test (OGTT) all animals were

10   fasted for 18-20 hours and were tail bled for baseline (time 0) measurements starting at approximately 10:00 AM. Subsequent to the tail bleed, animals were administered 89mg D-glucose (Sigma, St. Louis, MO) dissolved in 0.2ml distilled water (~2.2g/kg body weight) through a feeding needle (VWR, Plainfield, NJ). Blood was

15   drawn from the tail at 20, 60, and 210 minutes after the glucose administration. Serum was stored at -20oC until time of assay for blood glucose, insulin, free fatty acids, triglycerides (Linco Research Immunoassay, St Charles, MO) as previously outlined (Tonra, J.R., et al., 1999, Diabetes 48:588-594).

20

(2) Tissue sampling, homogenation and immunoprecipitation

In a separate group of experiments, mice were studied to examine the effect of Ax-15 treatment on receptor signaling components. After the indicated times and doses of Ax-15 (see

25   above), liver tissue was isolated and snap frozen for subsequent analysis. Tissue samples (100mg) were homogenized on ice in Buffer A (1% NP-40, 50 mM Hepes pH 7.4, 150mM NaCl, 1mM EDTA, 30mM sodium pyrophosphate, 50mM Sodium Fluoride, 0.5mM sodium

orthovanadate, 5µg/ml aprotinin, 5µg/ml leupeptin, 1mM PMSF) and centrifuged for 10 minutes at 14,000g. Lysate protein (2mg) was immunoprecipitated overnight at 4oC with either 5µl of anti-p(tyr) antibody (4G10) or anti-IRS-1 antibody coupled to Protein A

5   sepharose (Upstate Biotechnology, NY). The immunoprecipitates were washed three times with Buffer A, resuspended in standard Laemmli sample buffer and heated for approximately 5 minutes at 65°C. The protein samples were resolved by standard SDS-PAGE analysis on 6 or 8% precast gels and transferred to nitrocellulose

10   membranes (Novex, CA) using a Trans Blot system (Hoeffer Transblotter, Pharmacia, NJ). Nitrocellulose membranes were blocked with 5% BSA (for 4G10 blots) or 3% Blotto/0.5% BSA for at least 1 hour at room temperature and then incubated with the primary antibody overnight at 4oC. Antibodies used included anti-

15   p(tyr) 4G10 (1:5000; Upstate Biotechnology Inc); anti-IRS-1 and anti p85 (New England Biolabs, Beverley, MA).

(3) Immunohistochemistry

Animals to be assessed by immunohistochemistry were

20   perfused transcardially with 4% paraformaldehyde and the brains were removed and frozen until processed. Forty µm sections were cut at the level of the arcuate nucleus, washed in KPBS (potassium buffer saline, pH 7.2) and blocked for 20 minutes at room temperature (4 % normal serum in KPBS/0.4% Triton X100/1% Bovine

25   Serum Albumin, Fraction V, Sigma). The free floating sections were incubated overnight at 4oC with mouse anti-p(tyr) (4G10) at a 1:1000 dilution to detect p(tyr) protein, washed, then incubated with biotinylated horse anti-mouse antibody diluted in buffer

99

(KPBS/0.02% Triton X-100/1.0% BSA) at 1:1500 dilution followed by avidin-biotin peroxidase (1:500 in PBS; Vector Elite Kit, Vector Laboratories, Burlington, CA) both for 60 minutes and at room temperature. Between each step sections were washed thoroughly in PBS and the tissue-bound peroxidase was visualized by a diaminobenzidine (Sigma St Louis, MO) reaction mounted on gelatin-coated slides, dehydrated, and coverslipped.

Results

(1) Treatment of _db/db_ animals with daily Ax-15 causes a significantly greater weight loss than does caloric restriction. _db/db_ mice or their heterozygous litter mates (_db/?_) were given daily injections (s.c.) of either Ax-15 (0.1 or 0.3 mg/kg) or vehicle for 10 days. Food intake was restricted for a cohort of vehicle treated animals (Pair-fed) to the same amount ingested by the highest Ax-15-treated group. Figure 23 shows the results of this experiment. The mean group bodyweight +/- SEM (n=12) is reported for each day. Peripheral administration of Ax-15 (0.1 & 0.3 mg/kg/day for 10 days) produced a significant reduction in food intake and dose dependent reduction in bodyweight (BW). For the highest dose tested, the effect on BW was greater than attributable to caloric restriction (c.f pairfed vehicle _db/db_; PF) and was associated with a reduction in the mass of epididymal fat (by 25%) and liver (35%) with no effect on muscle mass.

(2) The effect of 10 day Ax-15 treatment on glucose tolerance in _db/db_ animals. An oral glucose tolerance test (OGTT) was performed on vehicle (open square), pairfed-vehicle

treated (filled diamond), and Ax-15 treated (0.1 mg/kg/day, open triangle; 0.3 mg/kg/day, filled triangle) *db/db* male mice and age-matched heterozygous *db/?* mice (filled circle). Figure 24 shows the results of this experiment. Each point represents the mean of at

5 least twelve animals ± SEM. There was a reduction in fasting plasma glucose (by 65%), insulin (by 53%) and NEFA (23%) compared to vehicle treated levels. Oral glucose tolerance tests revealed a dose dependent improvement in glucose tolerance, with the area under the curve significantly different from PF and vehicle controls.

10

(3) Treatment of *db/db* animals with daily low doses of Ax-15 causes a significant body weight loss. *db/db* mice were given daily injections (s.c.) of either Ax-15 (0.0125, 0.025 or

15 0.05 mg/kg) or vehicle for 10 days. The mean group bodyweight +/- SEM (n=6) is reported for each day. As shown in Figure 23C, body weight is reduced in a dose-dependent manner.

(4) The effect of 10 day low dose Ax-15 treatment o n

20 **glucose tolerance in *db/db* animals.** An oral glucose tolerance test (OGTT) was performed on vehicle (open square) and Ax-15 treated (0.0125, 0.025 or 0.05 mg/kg) *db/db* male mice. Each point represents the mean of at least six animals ± SEM. As shown in Figure 23D, plasma glucose is reduced in a dose-dependent manner,

25 with the 0.05 mg/kg dose exhibiting the greatest reduction is plasma glucose.

(5) Time course of effects of Ax-15 treatment. Time course of effects of Ax-15 treatment (0.3 mg/kg/day; filled triangle)

101

compared to vehicle treated (open square), pairfed-vehicle treated (filled diamond) on non-fasting serum blood glucose from *db/db* male mice. Each point represents the mean of at least six animals ± SEM 14 hour after the last injection. As shown in Figure 24, Ax-15

5   significantly reduces non-fasting serum blood glucose by the third day of treatment as compared to vehicle treated or pairfed-vehicle treated mice.

(6) Physiological consequences of 10-day Ax-15 treatment

10   **in *db/db* animals.** Figure 25A-25C shows the results of an experiment that was designed to evaluate the physiological consequences of 10-day Ax-15 treatment on db/db mice. Figure 25A: Fasting blood glucose concentrations were determined with serum from *db/db* male mice treated for 10 days with Ax-15 (0.1

15   mg/kg/day and 0.3 mg/kg/day, hatched bars) as compared to control groups, vehicle treated (open bar), pairfed-vehicle treated (hatched bar) and age-matched heterozygous *db/?* mice (stippled). Each bar represents the mean of at least eight animals ± SEM. Figure 25B Fasting insulin concentrations were determined on serum from *db/db*

20   male mice treated for 10 days with Ax-15 (0.1 mg/kg/day and 0.3 mg/kg/day, hatched bars) as compared to control groups, vehicle treated (open bar), pairfed vehicle-treated (hatched bar) and age-matched heterozygous *db/?* mice (stippled). Each bar represents the mean of at least eight animals ± SEM. Figure 25C: Fasting free fatty

25   acid levels were determined on serum samples from *db/db* male mice treated for 10 days with Ax-15 (0.1 mg/kg/day and 0.3 mg/kg/day, hatched bars) in comparison to control groups, vehicle treated (open bar), pairfed-vehicle treated (hatched bar) and age-matched

102

heterozygous *db/?* mice (stippled). Each bar represents the mean of at least eight animals ± SEM.

(7) The effects of Ax-15 treatment on insulin-stimulated p(tyr) immunoreactivity in the arcuate nucleus *of db/db* mice. Immunostaining of heterozygous *(db/?)* mice showed an increase in p(tyr) immunoreactive staining neurons of the arcuate nucleus (Figure 26B) following a 30 minute bolus of insulin (1 IU via the jugular vein) as compared to vehicle injected control level (Figure 26A). This result presumably reflects neurons in the arcuate nucleus that express insulin receptors and its substrates (*eg*. IRS-1), both of which are phosphorylated after insulin binding. Analysis of the insulin resistant/diabetic *db/db* mice (vehicle treated for 10 days) revealed a constitutively high p(tyr) immunoreactive staining pattern (Figure 26C) with no detectable change after insulin treatment (Figure 26D). Ten day Ax-15 treatment of *db/db* mice attenuated the high basal p(tyr) immunoreactivity (Figure 26E and 26G) and restored insulin p(tyr) responsiveness (Figure 26F and 26H).

(8) The effects of Ax-15 treatment on insulin-stimulated signaling in the liver of *db/db* mice. Figure 27A-27B shows the results of an experiment designed to evaluate the effects of Ax-15 treatment on insulin-stimulated signaling in the liver of *db/db* mice. Male *db/db* mice were treated for 10 days with either vehicle (lanes 7 & 8), pairfed to drug treatment levels (lanes 1 & 2) or treated with Ax-15 (0.1 mg/kg/day, lanes 5 & 6; 0.3 mg/kg/day, lanes 4 & 5). On the 11th day animals were anaesthetized with pentobarbital and injected with either saline (-) or 1 IU of regular insulin (+) via the portal vein. The liver was removed after 1 min,

103

and protein extracts were subjected to immunoprecipitation with an anti-p(tyr) specific antibody 4G10 followed by standard Western blot analysis with an antiserum to the p85 regulatory subunit of PI3-kinase (Figure 27A), IRS-1-specific antisera followed by

5　　Western blot analysis with an anti-p(tyr)-specific antibody (Figure 27B, upper panel), and an IRS-1-specific antiserum (Figure 27B, bottom panel).　Non-immune control immunoprecipitation (NI), no lysate control (NL), and 3T3-L1 lysate control for p85 (C) were run as immunoprecipitation and blotting controls.

10　　　Analysis of insulin action in peripheral tissues from Ax-15 treated mice indicated enhanced tyrosine phosphorylation (ptyr) of specific substrates (IRS-1) and increased p(tyr) associated PI3 kinase in response to an acute i.v. insulin bolus. Immunohistochemical assessment at the level of the arcuate nucleus

15　　in the CNS revealed that Ax-15 treatment attenuates the elevated basal p(tyr) levels seen in vehicle treated *db/db* and restores insulin-stimulated p(tyr).　These data suggest improved peripheral glucose tolerance and restoration of both peripheral and central insulin-dependent signaling events with Ax-15 treatment in animals

20　　that lack the functional long form of the leptin receptor (i.e. *db/db*).

These results indicate that Ax-15 has the ability to normalize glucose metabolism over and above the effect caused by weight loss alone, suggesting the utility of CNTF or its variants for the normalization of glucose metabolism in patients having abnormal

25　　glucose metabolism such as hyperinsulinemics, hypoglycemics, or diabetics, especially Type II or non-insulin dependent (NIDDM) diabetics.